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Award Number DAMD17-94-J-4179

TITLE: Her-2-/Neu and Breast Cancer

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REPORT DATE: July 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Final (8 Jul 94 - 7 Jul 99)

4. TITLE AND SUBTITLE
Her-2-/Neu and Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4179

6. AUTHOR(S)
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Doctor Gail Clinton

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oregon Health Sciences University
Portland, Oregon 97201-3098

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT

HER-2/*neu* is a potent oncogene that predicts poor outcome when overexpressed in ovarian cancer. The SKOV-3 ovarian carcinoma cell line, one of the only models for HER2-driven ovarian cancer, expresses a major uncharacterized 8 kb alternative HER-2 transcript. The aim of this study was to characterize the structure, determine the origin of the alternative sequence, and examine the possible role of the 8 kb alternative transcript in overexpression of the HER-2 gene. The structure of the 8 kb transcript was investigated using the polymerase chain reaction (PCR) and nucleotide sequencing of cDNA clones. PCR analysis of genomic DNA was used to assess the origin of the 8 kb transcript. The stability of the 8 kb mRNA was assessed by Northern blot analysis of RNA extracted from cells treated with transcriptional inhibitors. Similar 5'UTR and coding sequence but an extended 3'UTR was contained in the 8 kb compared to the well-characterized 4.5 kb HER-2 transcript. Genomic DNA had continuity between the novel 3'UTR sequence from the 8 kb transcript and adjacent HER-2 terminal exon sequence. The 8 kb transcript had a half-life of 13 h compared to 5.5 h for the 4.5 kb transcript ($p<0.01$). The 8 kb transcript is generated from alternative polyadenylation site usage rather than gene rearrangement. Since the 8 kb transcript contains alternative sequence found at the 3'end of the normal HER-2 gene, it could be expressed in other cells. Increased stability of the 8 kb transcript may confer a selective advantage for SKOV-3 cells by providing enhanced HER-2 expression.

14. SUBJECT TERMS
Breast Cancer
HER-2/*neu*, proto-oncogene, cDNA, 3'UTR, 5'UTR, RT-PCR, RACE, $p185^{HER-2/neu}$

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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7-8-99

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Introduction: Background and Significance

The HER-2/neu oncogene is the human homologue of the *neu* proto-oncogene, which was initially identified in rat neuroblastomas induced by the mutagen N-ethyl-nitrosylurea (Coussens et al., 1985; Yamamoto et al., 1986). The human HER-2/neu gene is located on the long arm of chromosome 17 and spans approximately 78 kilobases (kb) with at least 28 exons (Coussens et al., 1985). Like HER-2, the *neu* gene encodes a single membrane-spanning receptor tyrosine kinase, p185neu, with extensive homology to the epidermal growth factor receptor (EGF-R). Unlike HER-2, *neu* requires an activating mutation for its transforming capacity in the rat: a single base-pair mutation that results in a Valine to Glutamic acid substitution at amino acid residue 664 (V664E), which resides within the transmembrane region of the molecule (Bargmann et al., 1986). Introduction of a charged residue at this position was subsequently shown to confer constitutive tyrosine kinase activation upon rat *neu* (Bargmann & Weinberg, 1988). The orthologous mutation (V659E) in the human HER-2/neu gene product, p185HER-2, requires two base pair changes and has not been found in human tissues (Hynes & Stern, 1994; Suda et al., 1990).

The HER-2 gene is transcribed into a 4.5 kb HER-2 messenger RNA (mRNA) and 185 kilodalton (kDa) protein product, p185HER-2 (Coussens et al., 1985), which is developmentally expressed and is essential (Lee et al., 1995), yet its normal function is entirely unknown (Tzahar & Yarden, 1998). HER-2/neu gene disruption causes early embryonic lethality (E9-10) in mice, and is required for normal development of neural tissues and ventricular trabeculation of the myocardium (Lee et al., 1995). HER-2/neu is expressed abundantly in many human fetal tissues and displays ubiquitous low levels of expression in adult human tissues (Press et al., 1990), with the exception of some hematopoetic cell lineages that do not express detectable levels of HER-2 (Hynes & Stern, 1994).

Class I Receptor Tyrosine Kinases

The HER-2/neu gene product is a member of the EGF-R family (class I) of receptor tyrosine kinases (RTK). There are four homologous members of the EGF-R family: HER-1 (erbB-1, EGF-R), HER-2 (neu, c-erbB-2), HER-3 (c-erbB-3), and HER-4 (Hynes & Stern, 1994). EGF-R

is the prototype of class I RTKs with the following structural features (Ullrich & Schlessinger, 1990). The extracellular domain (ECD) confers ligand binding and is divided into four subdomains (I-IV)(van der Geer et al., 1994). Subdomains I and III are implicated in low- and high-affinity receptor-ligand interactions, respectively, since sites within these regions were shown to be involved in EGF-R bivalent ligand binding (Lax et al., 1988; Tzahar et al., 1997; Summerfield et al., 1996; Lax et al., 1991). Subdomains II and IV are rich in cysteine residues with a highly-conserved distribution, and are thought to provide the structural features of the ligand-binding pocket (Lax et al., 1991). The ECD is connected to the intracellular domain by a single transmembrane domain (van der Geer et al., 1994). The cytoplasmic domain is composed of a highly conserved tyrosine kinase catalytic site that is responsible for receptor transphosphorylation and substrate phosphorylation (Ullrich & Schlessinger, 1990). In addition, the carboxyl-terminus contains several tyrosine autophosphorylation sites of unique sequence that mediate activation state-dependent signaling via second messenger association with phosphorylated sites (van der Geer et al., 1994; Ullrich & Schlessinger, 1990). Among known intracellular second messengers of EGF-R signaling are the phosphotyrosine binding (PTB)-domain proteins, phosphatidyl inositol-3 kinase (PI-3 K) and phospholipase Cγ (PLCγ), and src-homology domain-containing (SH2) proteins, such as Shc and Grb2, which mediate signaling through the mitogen-activated protein (MAP) kinase cascade (Cohen et al., 1995; Seger & Krebs, 1995; Kavanaugh et al., 1994; Ming et al., 1994).

Activation of Class I RTKs

In addition to sharing structural features, the EGF-R family members are activated by a common mechanism. Activation is initiated by receptor dimerization that is typically induced by ligand binding to cognate receptor monomers with one-to-one stoichiometry (Tzahar et al., 1997; Heldin & Ostman, 1996; van der Geer et al., 1994; Woltjer et al., 1992; Ullrich & Schlessinger, 1990). Receptor dimerization is an absolute requirement for activation of the tyrosine kinase catalytic domain (Weiss & Schlessinger, 1998; Qian et al., 1995). Catalytic activation is thought to be mediated by a conformational change in receptor monomers induced by ligand binding and subsequent dimer formation (Weiss & Schlessinger, 1998; Tzahar et al., 1997; Dougall et al., 1994). Dimers can be homomeric or heteromeric among all four members of the EGF-R family (Earp et al., 1995; Carraway & Cantley,

1994). Indeed, all ten possible dimers have been shown to occur in a hierarchical nature, with p185HER-2 as the overall preferred dimer partner (Graus-Porta et al., 1997; Tzahar et al., 1996).

Despite strong similarities, several distinctions exist between EGF-R family members. EGF-R (HER-1) and HER-4 are activated by their cognate ligands in the conventional manner (Beerli & Hynes, 1996; Riese et al., 1996). HER-3, however, shares homologous structure and specifically binds NDFs, inducing rapid dimerization, but is kinase inactive (Zhang et al., 1996; Wallasch et al., 1995; Guy et al., 1994). Conversely, HER-2 is an orphan receptor to which no ligand that binds directly with high affinity has yet been identified, despite intense investigations (Tzahar & Yarden, 1998; Pinkas-Kramarski et al., 1998, 1996; Peles & Yarden, 1993; Peles et al., 1993, 1992). Thus, without a known activation-inducing ligand, HER-2 homodimer function in signaling has been difficult to study. To date, the signaling activation of HER-2 has mainly been investigated with regard to heterodimer signaling, as HER-2 has been shown to undergo heterodimerization in a ligand-inducible manner with cognate co-receptors, especially the kinase-inactive HER-3 (Tzahar et al., 1996; Zhang et al., 1996; Earp et al., 1995; Wallasch et al., 1995).

EGF-like Ligands

Regulation of EGF-R family member activity is complex, owing to the discovery of several EGF family ligands and the fact that these receptors form homodimers as well as heterodimers. All known mammalian EGF family ligands function to stimulate dimerization of their cognate receptors (Tzahar & Yarden, 1998). The motif responsible for activation is an EGF domain, comprising six cysteines and a few other essential residues with a characteristic, conserved distribution (Groenen et al., 1994). It has recently been shown that EGF-R family ligands are bivalent (Tzahar et al., 1997). The amino-terminus of their EGF-like domain binds to subdomain III of the receptor with high affinity and narrow specificity (Tzahar et al., 1997; Summerfield et al., 1996). The carboxyl-terminal region of the ligand binds to a second receptor, the dimer partner or co-receptor, with low affinity and broad specificity to a site that may be within subdomain I of the co-receptor (Woltjer et al., 1992; Lax et al., 1991, 1990).

Specificity for their cognate receptors serve to classify ligands into three groups. Epidermal growth factor (EGF), transforming growth

factor alpha (TGFα), and heparin-binding EGF-like growth factor (HB-EGF) activate EGF-R with nM affinity (Higashiyama et al., 1991; Savage et al., 1972). Betacellulin (BTC) is a ligand for both EGF-R and HER-4 (Beerli & Hynes, 1996; Riese et al., 1996). Neu differentiation factors (NDF), or heregulins, exist in several alternatively spliced forms and bind to both HER-3 and HER-4 with high affinity (Tzahar et al., 1997; Peles & Yarden, 1993; Plowman et al., 1993). Recently, neuregulin-3 was identified as a HER-4 ligand in neural tissue, where this receptor is endogenously expressed (Zhang et al., 1997). In addition to ligand-mediated stimulation of RTKs, their activation can also occur in response to stress, such as hyperosmotic shock and UV irradiation by an unknown mechanism (Weiss et al., 1997).

Although there is a growing number of known activating ligands, few natural inhibitors or antagonists have been described. Among them, only one, the Drosophila Argos, is a class I RTK antagonist (Howes et al., 1998). Argos is structurally related to the activating EGF-R family ligands in that it contains a single EGF domain and is secreted (van de Poll et al., 1997; Schweitzer et al., 1995). Argos is postulated to exert its antagonistic effect by competing with activating ligands for binding, thereby inhibiting EGF-R dimerization (Schnepp et al., 1998). Angiopoietin-2 is the other known extracellular inhibitor of a mammalian RTK, the Tie-2 endothelial receptor, to which it binds, but fails to induce activation (Maisonpierre et al., 1997). Much effort has been invested in understanding the mechanisms underlying ligand binding and activation in order to facilitate designing inhibitors, since antagonists of EGF-R family members and other class I RTKs have potential therapeutic utility (Groenen et al., 1994).

Oncogenic Activity of HER-2

Gene transfer studies have shown HER-2/neu to be the single most potent oncogene in its unaltered form. Overexpression alone of the wild-type HER-2/neu cDNA, encoding p185, confers malignant transformation (DiFiore et al., 1987; Hudziak et al., 1987). This occurs in the apparent absence of an activating ligand (Dougall et al., 1994; Rodrigues & Park, 1994; Segatto et al., 1988). In addition, transgenic mice engineered to overexpress "wild-type" human p185HER-2 develop metastatic mammary tumors (Guy et al., 1992; Suda et al., 1990). This is in contrast to any other RTK and to rat p185neu, which require either the presence of ligand or activating mutations to induce a malignant phenotype (Cohen et al., 1996;

Dougall et al., 1994; Weiner et al., 1989; Bargmann et al., 1986; Hung et al., 1986).

EGF-R family members, especially p185HER-2, have been implicated in many human cancers (Hynes & Stern, 1994). HER-2/neu overexpression has been found in several human adenocarcinomas, and, most notably, in up to 30% of human breast and ovarian carcinomas, where its overexpression confers poor prognosis (Ross et al., 1998; Press et al., 1990; Slamon et al., 1989, 1988, 1987). HER-2 expression may have an important role in many other human tumors of epithelial origin as well, including colon, prostate, non-small cell lung cancer, cervical, esophageal, and oral squamous cell carcinoma (Cohen et al., 1989; Arai et al., 1997; Kern et al., 1990; Hynes & Stern, 1994; Mitra et al., 1994).

Significant evidence exists that HER-2/neu is a potent oncogene in human adenocarcinomas. In both breast and ovarian cancer, where HER-2 has been most widely studied, increased p185HER-2 expression levels resulting from HER-2 gene amplification predict a decreased survival time in a dose-dependent manner(Slamon et al., 1989, 1988, 1987). p185HER-2 overexpression in breast cancer predicts a 9.5-fold increased relative risk for tumor recurrence (Press et al., 1990). In addition, HER-2/neu overexpressing breast and ovarian tumors display decreased responsiveness to adjuvant chemotherapy and to endocrine therapy, such as tamoxifen (Pegram et al., 1997; Felip et al., 1995; Benz et al., 1993).

Further evidence of the oncogenic effects of HER-2 have been gained by abrogating HER-2 expression at the surface of human tumor cells, the overwhelming effect of which is tumor regression. Downregulation of p185HER-2 expression by several approaches has confirmed this. Conditional HER-2 expression using a tetracycline-repressible promoter can regulate tumorigenesis (Fruendieb et al., 1997; Baasner et al., 1996; Kistner et al., 1996); ribozymes (Juhl et al., 1997) and antisense oligonucleotides (Pegues & Stromberg, 1997) have been used to selectively degrade HER-2 mRNA; coexpression of adenovirus EIA has been used to decrease HER-2 translation and synthesis (Yu et al., 1993); single-chain intracellular antibodies that bind nascent HER-2 monomers and trap it in the endoplasmic reticulum have been effective (Deshane et al., 1995; Beerli et al., 1994); forced expression of dominant negative receptor isoforms have been used to bind the receptor at the cell surface and block its dimerization and activation (Qian et al, 1994); and extracellular

domain-specific monoclonal antibodies have been used to downregulate p185HER-2 cell surface expression by inducing internalization (Rodriguez et al., 1993; Hudziak et al, 1989).

Mechanisms for Enhanced Potency

The current model describing the oncogenic potency of HER-2 states that HER-2 gene amplification leads to increased transcription of the 4.5 kb mRNA encoding p185HER-2 and results in cell surface overexpression. Overexpression favors dimerization and, thus, tyrosine kinase catalytic activation (Dougall et al., 1994; Samanta et al., 1994; Lonardo et al., 1990). Activation by autophosphorylation and transphosphorylation leads to enhanced signaling for growth and tumor cell proliferation, and further may induce an invasive, anchorage-independent phenotype (Xu et al., 1997; Yu et al., 1994; Yu et al., 1993).

Possible mechanisms underlying the oncogenic potency of p185HER-2 involve its high intrinsic kinase activity in auto- and transphosphorylation. In addition, p185HER-2 displays enhanced ligand-independent homodimerization and activation with increased cell surface expression (DiFiore et al., 1987; Hudziak et al., 1987). Although the possibility of an as-yet-uncharacterized activating ligand is not ruled out, this seems unlikely since heterologous HER-2 overexpression induces a transformed phenotype, which appears to be ligand-independent (Segatto et al., 1988; Hudziak et al., 1987).

Another possible mechanism for the oncogenic potency of HER-2 is by heterodimerization. As the preferred heterodimer partner, HER-2 displays synergistic transforming activity when co-expressed with either EGF-R (Kokai et al., 1989) or HER-3 (Alimandi et al., 1995; Wallasch et al., 1995). The preferential interaction with p185HER-2 is suggested to occur through a low-affinity, promiscuous ligand-binding site located within its subdomain I, which interacts with a broad specificity binding site within the carboxyl-terminus of the ligand's EGF-like domain (Tzahar et al., 1997). According to this model, ligand bivalence promotes heterodimer interaction by a high affinity interaction with its primary receptor and a promiscuous, low-affinity interaction with preference for p185HER-2 (Graus-Porta, 1997; Tzahar et al., 1997, 1996). Heterodimerization enhances RTK signaling and proliferative effects by deceleration of ligand dissociation from heteromeric receptor complexes and by enhanced recycling to the cell surface, rather than receptor degradation

(Lenferink et al., 1998; Huang et al., 1990). This latter effect is thought to occur by facilitating dissociation of receptor heterodimer-ligand complexes in the early endosome, as opposed to the late endosome or lysosome, where receptor constituents are destined to the degradation pathway.

Furthermore, HER-2 has been postulated to increase the invasive phenotype via interaction of p185HER-2 with beta-catenin. Activated p185HER-2 has been shown to phosphorylate the carboxyl-terminus of beta-catenin and induce its dissociation from E-cadherin in adhesion complexes (Ochiai et al., 1994) which mediate the tight cell-to-cell junctions of epithelial cell layers. Dissociation of beta-catenin from adhesion complexes is thought to weaken cell-to-cell adhesions (Adams et al., 1996). Studies conducted in breast carcinoma cells using forced expression of dominant-negative amino-terminal deletion mutants of beta-catenin in an invasion assay support the hypothesis that this HER-2-mediated phosphorylation event might initiate an invasive phenotype (Shibata et al., 1996). Further, it has been proposed that this event may facilitate progression to anchorage-independence and metastasis by allowing HER-2 overexpressing tumor cells to escape the epithelial monolayer.

Mechanisms for Overexpression of HER-2 in Human Cancer

Accumulated evidence supports a role in human tumorigenesis for overexpression, as the primary aberrance, of the unaltered HER-2/neu gene. Proposed mechanisms for increased HER-2 expression include gene amplification, transcriptional, and post-translational upregulation (Kraus et al., 1987).

Gene Amplification

HER-2 gene amplification has been detected in 20-25% of human breast and ovarian cancers, and likely represents a major mechanism for HER-2 overexpression in human tumors (Zhang et al., 1989). Increase in gene copy number occurs by an unknown mechanism resulting in apparent tandem duplication of the unaltered gene.

Enhanced mRNA Levels

HER-2 mRNA accumulation is observed in conjunction with gene amplification, but to a greater extent than can be accounted for by increased gene copy number alone, in many cell lines that exhibit HER-2 overexpression (Hynes & Stern, 1994). In several breast and ovarian carcinoma cell lines, including SKBR3, BT474, and SKOV-3,

the HER-2 transcript is expressed at up to 140-fold the level detected in nontumorigenic cells (Hollywood & Hurst, 1993; Tyson et al., 1991). Although both transcriptional and post-transcriptional mechanisms for mRNA accumulation are possible (Kornilova et al., 1992), upregulation of HER-2 mRNA has only been reported to occur as a result of increased transcription initiation rate (Hollywood & Hurst, 1993).

Clinical Utility of the HER-2 Oncogene

The high incidence of p185HER-2 overexpression and its association with poor prognosis suggest that it should be an important target of cancer therapy. Moreover, since it is expressed at the cell surface at abnormally high levels in tumor as opposed to normal cells, it provides a therapeutic target at the molecular level. Thus, p185HER-2 overexpression has led to efforts at developing anti-cancer therapeutics that target the ECD (Pelgram et al., 1998; Baselga et al., 1996; Rodriguez et al., 1993; Hudziak et al, 1989).

One example is a humanized recombinant monoclonal antibody, Herceptin (Genentech), which is targeted toward the ECD (Baselga et al., 1996). Herceptin recognizes an epitope that is located between residues 529 and 625 (Michael Shepard, personal communication with JPA). Herceptin causes down-regulation of p185HER-2 at the cell surface and may induce its degradation (Baselga et al., 1996; Hudziak et al., 1987). In addition, Heregulin induces antibody-dependent cellular cytotoxicity (ADCC) by activating the complement pathway and immune cells *in vivo* (Pegram et al., 1997). Herceptin treatment alone showed a 12-16% response rate in patients with HER-2-overexpressing metastatic breast cancer in phase II (Baselga et al., 1996) and phase III clinical trials (Pegram et al., 1997), and it has been shown to increase effectiveness of adjuvant chemotherapy in HER-2 overexpressing cancers by two-fold, eliciting a 24% response rate (Pegram et al., 1998). Herceptin has recently received Federal Drug Administration (FDA) approval for use as an anti-cancer therapeutic in breast carcinoma patients. However, Herceptin is less effective than was originally predicted, given that 30% of breast cancers overexpress p185HER-2. Since 100% of patients entered in the Herceptin clinical trials had HER-2 overexpressing breast cancer, this level of response translates to only about 4% of the total breast cancer patient population.

One explanation for the low level of effectiveness that was addressed in the Herceptin clinical trials is the presence of a soluble HER-2 ECD that is proteolytically cleaved at the surface of tumor cells, shedding the target that Herceptin is directed against into the serum (Lin & Clinton, 1990). The presence of the soluble ECD can be detected in breast cancer patients, occurring at serum concentrations of up to 5 μg/ml, and correlates with tumor load in metastatic disease (Brodowicz et al., 1997a; Molina et al., 1996; Kandl et al., 1994). This soluble HER-2 ECD contains the recognition epitope of Herceptin and neutralizes the antibody's cytotoxic effects (Baselga et al., 1996; Brodowicz et al., 1997b). Indeed, it was reported that the titer of circulating Herceptin quickly fell below therapeutic levels in patients with detectable HER-2 ECD in serum (Pelgram et al., 1998; Baselga et al., 1996). Moreover, Herceptin response inversely correlated with serum levels of the soluble HER-2 ECD: patients whose tumors progressed during the trials demonstrated a concomitant rise in serum HER-2 ECD levels (Pelgram et al., 1998).

Another potential method of specifically targeting HER-2 overexpressing tumor cells is ligand- or antibody-mediated toxin delivery to these cells (Rodriguez et al., 1993). Unfortunately, anti-HER-2 therapeutic design based on the current model, stating that overexpression of p185HER-2 is the primary aberrance, has proven surprisingly limited in effectiveness against HER-2 overexpressing breast cancers.

Nature of the Problem & Purpose of the Present Work

HER-2 as a Prognostic Marker

The clinical utility of HER-2 as a prognostic marker, based on the expression level of p185HER-2 in tumor tissues, is limited. Numerous clinical trials have been conducted and, cumulatively, it has been determined that HER-2 overexpression predicts poor prognosis only in lymph node positive disease (Singleton & Strickler, 1992). Thus, lymph node status remains a better prognostic indicator (Hynes & Stern, 1994; Tandon et al., 1989). There remains a need for identification of an early tumor marker that will be useful in node-negative disease.

Alternative HER-2 gene products

HER-2 function has mainly been studied based on biochemical and functional studies of the p185HER-2 product. Alternative mRNA transcripts of the HER-2 gene have been reported, although their functions have not been determined. A truncated transcript of 2.3 kb has been identified in a gastric carcinoma cell line, MKN7, was subsequently cloned from cDNA libraries of two HER-2-overexpressing breast carcinoma cell lines, BT474 and SKBR3, and was also detected in seven other carcinoma cell lines by the ribonuclease protection assay (Scott et al., 1993). Although the protein product of this transcript is predicted to be secreted, since it lacks the transmembrane-anchoring domain, it was detected only in the endoplasmic reticulum of cells. The 2.3 kb truncated transcript results from read-through of a consensus splice donor site of an intron located 5' to the transmembrane domain-encoding exon. This intron contains a consensus polyadenylation signal. This process of alternative transcript generation is termed internal polyadenylation.

Alternative transcripts of the HER-3 gene have also been identified. Four alternative HER-3 transcripts, detected in several cell lines by ribonuclease protection assay, result from intron retention with internal polyadenylation, similar to the mechanism proposed for generation of the 2.3 kb alternative HER-2 transcript (Katoh et al., 1993; Scott et al. 1993). Furthermore, they were shown to encode stable protein products when transfected into Chinese hamster ovary (CHO) cells as expression cDNA (Lee & Maihle, 1998). However, these alternative HER-3 gene products have not yet been ascribed a function.

Characterization of an Alternative Form of HER-2/neu Oncogene Expression

This work describes the structure and function of an alternative product of the HER-2 gene. The overlying hypothesis is that alternative products are involved in the normal and malignant function of the HER-2.

An alternative HER-2/neu transcript of 8 kb has been reported in the HER-2-overexpressing ovarian carcinoma cell line, SKOV-3 (Jones et al., 1994; Karlan et al., 1994; Lichtenstein et al., 1990). This transcript appears to represent the major HER-2 transcript in these

cells (i.e., it is two- to three-fold more abundant than the 4.5 kb mRNA). It has been proposed that the transcript results from a gene rearrangement in SKOV-3. Yet, despite the fact that the alternative 8 kb HER-2 transcript had not been characterized, SKOV-3 have been used repeatedly as a model for HER-2-driven ovarian cancer. This work describes the characterization of the alternative 8 kb HER-2 transcript.

The human ovarian carcinoma cell line, SKOV-3, exhibits 8-fold amplification of the HER-2 gene (Karlan et al., 1994; Lichtenstein et al., 1990) and overexpresses both HER-2 mRNA and p185HER-2 by greater than 140-fold relative to most normal cells. Despite remarkably high levels of p185HER-2 in SKOV-3 cells, expression of even more p185HER-2 at the cell surface may further promote malignant growth. This was suggested by the increased p185HER-2 in SKOV-3 cells, selected, from early intraperitoneal metastases in nude mice that displayed stable enhanced metastatic potential (Yu et al., 1993). In addition to increased metastatic potential, the increased p185HER-2 expression correlated with enhanced growth rate of SKOV-3 cells. The additional increase in p185 occurred without evidence of further HER-2/neu gene amplification, suggesting transcriptional or post-transcriptional mechanisms (Yu et al., 1993). Indeed, HER-2 transcription is upregulated in the breast carcinoma cell lines BT474, SKBR3, and ZR75-1 (Hollywood & Hurst, 1993) and post-transcriptional mechanisms for increased HER-2 mRNA and protein expression have been found in HC11 (Kornilova et al., 1992) and in SKOV-3 cells (Karlan et al., 1994; Jones et al., 1994). Therefore, these studies suggest that, in addition to gene amplification, post-transcriptional mechanisms are important for upregulation of HER-2 mRNA levels in carcinoma cells.

In addition to the well-characterized 4.5 kb transcript, alternative HER-2 transcripts of unknown significance have been identified in both human tumors and tumor cell lines (Hung et al., 1992; Karlan et al., 1994; Lichtenstein et al., 1990; Jones et al., 1994; Scott et al., 1993; Fujimoto et al., 1995). SKOV-3 cells, which produce the highest level of p185HER-2 of any carcinoma cell line and are frequently used as a model for studies of HER-2 overexpression, also express an abundant mRNA species of approximately 8 kb (Karlan et al., 1994; Lichtenstein et al., 1990; Jones et al., 1994). While the mechanism underlying generation of this alternative transcript has not been determined, based on the presence of a polymorphism in

the HER-2 gene in SKOV-3 cells, it has been suggested that the 8 kb transcript results from a gene rearrangement (Hung et al., 1992).

In this study, we present evidence that the alternative HER-2 transcript contains similar 5'UTR and coding sequences to those of the 4.5 kb transcript. However, it differs in the length of its 3'UTR, which extends by approximately 3.5 kb from the 3'UTR found in the 4.5 kb transcript. Additionally, the 8 kb transcript displays increased stability, which could account for its overexpression. Our data support the possibility that the alternative 8 kb HER-2/*neu* transcript could confer increased p185HER-2 expression in SKOV-3 cells.

My work supports the overlying hypothesis that disruption of the normal regulation of HER-2 gene expression is the primary aberrance leading to HER-2-mediated transformation. Moreover, alternative HER-2 gene products may be involved in tumor development and progression. Defining the role of alternative products within the normal expression array of the HER-2 gene and in tumor models might help elucidate the discrepancies of the current model.

Body

An Alternative HER-2/*neu* Transcript of 8 kb has an Extended 3'UTR and Displays Increased Stability in SKOV-3 Ovarian Carcinoma Cells

by

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(manuscript in press for the Journal *Gynecologic Oncology*, 1999)

MATERIALS and METHODS

Cell culture. Human breast and ovarian carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD) maintained in either DMEM (SKBR3 and SKOV-3) or RPMI (BT474), supplemented with 10% fetal bovine serum and 0.05% gentamycin, as described (Hua et al., 1995). All media and supplements were obtained from GIBCO BRL (Life Technologies, Gaithersburg, MD). Unless specified, all other chemicals were purchased from Sigma.

RNA extraction. Cells at 80-90% confluence in 15 cm plates were extracted with TriReagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's protocol. RNA was resuspended in RNA sample buffer (50% formamide, 1.5% formalin, 0.1% SDS, 1mM EDTA, 10mM Tris pH 7.4) for Northern blotting, or in 10mM Tris-EDTA, pH 8.0, for mRNA fractionation and reverse transcription.

Isolation of the 8 kb HER-2/neu transcript for sequence analysis Poly-A+ RNA was selected from SKOV-3 cells using the Oligotex mRNA kit (Qiagen). 8 kb mRNA was fractionated from the 4.5 kb mRNA by electroelution from gels by a modification of a previously described method (Harty & O'Callaghan, 1991). Briefly, 0.1 mg SKOV-3 mRNA was electrophoresed on denaturing 3% formalin, 0.7 % agarose gels and gel regions containing mRNA of ~8 kb and ~4-5 kb were excised. The mRNA was electroeluted from the gel slices into dialysis tubing and formaldehyde was removed by dialysis against deionized water. The electroeluted mRNA was used as template for reverse transcription.

Polymerase chain reaction Fractionated mRNA was reverse-transcribed into first strand cDNA using GIBCO BRL (Life Technologies) Superscript RTII System with either random hexamers (for 5'RACE) or oligo-dT12-18 (for 3'RACE and for coding region amplifications). First strand cDNAs were used as template for PCR using HER-2 sequence-specific primers (GIBCO BRL, Life Technologies) and the Expand High Fidelity PCR System (Boerhinger Mannheim) with 2.5 mM MgCl2, 5 mM of each primer, and 200 mM dNTPs. Cycling parameters were: 30 cycles of 94° C for 30"; 58° C for 45"; 68° C for 3'.

For coding region amplification, a forward primer (5'-TGAGCACCATGGAGCTGGC-3') identical to nt 143-161 of HER-2 cDNA and reverse primer (5'-TCACACTGGCACGTCCAGACC-3') complimentary to nt 3898-3918 of HER-2 cDNA were used to amplify a 3.5 kb HER-2 fragment corresponding to the entire coding sequence (Coussens et al., 1985) of HER-2 cDNA (i.e., from start to stop codons).

Numbering of nucleotide and amino acid residues is according to Coussens et al. (1985).

For 5'RACE, single-stranded (or first strand) cDNAs synthesized from 8 kb and 4-5 kb fractionated mRNA were tailed with poly-deoxyadenosine using terminal deoxytranferase (Boehringer Mannheim). Oligo-dT12-18 was used to prime from the newly-synthesized poly-dA tail and a HER-2 sequence-specific reverse primer (5'-GGTGCACACTTGGGTGCTCG-3') complimentary to nucleotides 212-231 of HER-2 cDNA (Coussens et al., 1985) were used in the PCR.

For 3'RACE, first strand cDNAs were amplified using a forward primer identical to nt 3881-3898 of HER-2 cDNA (5'-AGAACCCAGAGTACCTGG-3'), and oligo-dT 12-18 as a reverse primer.

For PCR analysis of genomic DNA, the region spanning the polyadenylation signal sequence utilized in the 4.5 kb HER-2 transcript (Coussens et al., 1985) was amplified from SKOV-3, BT474, SKBR3, and normal human genomic DNA (a gift from Dr. Mike Litt, Oregon Health Sciences University), using a forward primer identical to nucleotides 4301-4323 of HER-2 cDNA and a reverse primer complimentary to unique sequence located approximately 100 bp downstream from the putative polyadenylation signal. DNA was prepared according to the procedure of Strauss (1998) and the PCR was performed for 25 cycles with cycling parameters of 94° C for 30"; 62° C for 30"; 72° C for 60".

Construction of a cDNA library from size-fractionated SKOV-3 mRNA Fractionated 8 kb mRNA was used as a template for cDNA library construction using a lZIPLOX kit with EcoR1 adaptors per manufacturer's protocol (GIBCO BRL, Life Technologies). Phage were packaged at room temperature for 1 h using a lamda phage packaging kit (Stratagene, La Jolla, CA). Complete phage were then used to infect Y1090 competent cells and the library was plated at ~25,000 pfu per 15 cm plate. Plates were incubated at 37° C for 16 h and screened using GeneScreen Plus Hybridization filters (NEN Life Sciences). Nucleic acids, fixed to membranes by UV crosslinking, were prehybridized for 2 h at 42° C in 30% formamide, 5M NaCl, and 1% SDS with 10 mg/ml of herring sperm DNA as blocking agent. A probe consisting of 108 cpm γ-(^{32}P)ATP end-labelled oligonucleotide, which is identical to nucleotides 4258-4276 of HER-2 cDNA (1), was hybridized with filters for 24 h at 42° C. Filters were then washed in several changes of 0.2XSSC at 55° C and exposed to film. DNA was eluted from positive clones and used to infect D12S cells, plated on LB agar containing 30 mg/ml kanamycin, 0.1 mg/ml IPTG and

0.015% X-gal. lZipLox plasmid DNA (pZL) was purified and sequenced in the Vollum Core Sequencing facility (Portland, Oregon) using universal m13 forward and reverse primers.

Northern blotting was performed as described previously (Hua et al., 1995) using 2.5 mg/lane of poly-A+ RNA. Hybridization was with 107 cpm of random primed $\alpha(^{32}P)$dCTP-labelled probe of either a 570 bp Xho1-EcoR1 fragment (5' coding region-specific) from the HER-2 expression vector p9002 (Applied BioTechnologies) or a 280 bp HincII cDNA fragment of the unique sequence obtained from an SKOV-3 cDNA library clone, synthesized using a Random Prime DNA Labelling Kit (Boehringer Mannheim). Blots were analyzed by phosphorimaging (Molecular Dynamics).

RESULTS

The alternative 8 kb transcript contains sequences from the 4.5 kb transcript. Northern blot hybridization studies revealed expression of an abundant aberrant HER-2 transcript of 8 kb in SKOV-3 cells (Fig.1). This alternative transcript is polyadenylated, since it was more abundant in poly-A+ selected RNA compared to total RNA. To assess the sequence content of the alternative HER-2 transcript, Northern blots were hybridized with probes corresponding to 5' coding, 3' coding and 3'UTR sequences contained in the 4.5 kb transcript. No difference was detected in the hybridization efficiencies of the different probes with the 8 kb and 4.5 kb transcripts, suggesting that the 8 kb transcript contains a single copy of the HER-2 coding sequence.

The coding sequence and the 5'UTR of the 8 kb and 4.5 kb transcripts are the same size. To examine whether the entire HER-2 coding sequence was contained in the 8 kb transcript, PCR amplification of the 3.5 kb open reading frame of the HER-2 cDNA sequence was targeted using forward and reverse primers flanking the translation initiation and termination codons. Amplification by RT-PCR of SKOV-3 mRNA of ~ 8 kb fractionated by size as described in Methods indicated that the 8 kb transcript contained HER-2 coding sequence of the same size (Fig. 2A, left panel), the identity of which was verified by Southern blot analysis (Fig. 2A, right panel). Sequence identity was further verified by restriction digest and sequence analysis of the PCR products (data not shown), indicating similar sequence content with identical sequence near the initiation and termination codons.

The 5'UTR of the 8 kb and 4.5 kb HER-2 transcripts are the same size. To further examine the structure of the 8 kb alternative transcript, we compared the 5'UTR of the 4.5 and 8 kb HER-2 transcripts using RT-PCR and 5' Rapid Amplification of cDNA Ends (5'RACE). HER-2 5'RACE revealed an amplification product of about 250 bp from both the 4.5 kb and 8 kb cDNA fractions (Fig. 2B, left panel), the identity of which was verified by Southern blot hybridization (Fig. 2B, right panel). This is the size expected for the normal 5'UTR of the 4.5 kb transcript, indicating no differences in the size of the 5'UTR of the 8 kb and 4.5 kb transcripts.

The 3'UTR of the 8 kb HER-2 transcript is extended and contains unique sequence. 3' RACE consistently yielded nonspecific amplification products of heterogeneous sizes generated from the 8 kb mRNA, while a PCR product of the expected size (~650 bp) was easily obtained from the 4.5 kb mRNA fraction and from reverse-transcribed mRNA from other cells (Fig. 2C). Since the same template was used to amplify the coding sequence and 5'UTR of the 8 kb mRNA, the alternative transcript appears to differ in its 3'UTR.

To directly investigate the sequence of the 3'UTR, an SKOV-3 cDNA library was constructed using fractionated SKOV-3 mRNA enriched in transcripts of ~8 kb as starting material. The SKOV-3 cDNA library was screened using a probe for the HER-2 3'UTR. Six of seven clones contained partial coding and 3'UTR sequence of HER-2 cDNA consistent with the reported sequence for the 4.5 kb transcript (Coussens et al., 1985). A single clone of approximately 7 kb was isolated and found to contain HER-2 3'coding sequence and 3'UTR identical to nucleotides 3138 to 4531, including the termination codon at nt 3916-3918. The sequence of this clone diverged 13 nt following the polyadenylation signal, which is reported to be utilized in generation of the 4.5 kb transcript (Coussens et al., 1985). Partial sequence of this clone is shown in Figure 3, indicating the polyadenylation signal (underlined) which is identical to that reported for the 4.5 kb transcript (Coussens et al., 1985), and additional sequence that continues for another 2 kb. The novel sequence contains multiple termination codons in all six reading frames, further indicating that it is untranslated. The extended sequence is unique with no homologous sequence identified in GenBank and Blast (Entrez) database programs. .

To verify that this sequence was specific to the 8 kb HER-2 transcript, a Northern blot of SKOV-3 and T47D mRNA was probed with the unique sequence (Fig. 4B) and with 5' HER-2 coding sequence, as a control (Fig. 4A). A 280 bp HincII fragment of the unique 3'UTR sequence hybridized only with the 8 kb transcript in

SKOV-3 but not with mRNA from T47D, a carcinoma cell line that does not express the 8 kb alternative HER-2 transcript. Therefore, this sequence is uniquely contained in the alternative 8 kb HER-2 transcript expressed in SKOV-3.

The extended 3'UTR of the 8 kb HER-2/neu transcript results from read-through of polyadenylation signals. Southern blot analysis has revealed the presence of an EcoR1 restriction fragment length polymorphism (RFLP) in the HER-2 gene (Fig.5 and refs.: Hung et al., 1992; Karlan et al., 1994), which has led to suggestions that the 8 kb transcript is generated as a result of gene rearrangement in SKOV-3 cells (Hung et al., 1992). To determine whether the alternative HER-2 transcript is a product of gene rearrangement or of alternative splicing, genomic DNA was amplified by PCR from SKOV-3, BT474, SKBR-3, and normal human genomic DNA using primers that span the polyadenylation signal from the 4.5 kb HER-2 3'UTR sequence (Coussens et al., 1985) and the unique 3'sequence obtained from the cDNA clone of the 8 kb transcript. BT474 and SKBR-3 cells have HER-2 gene amplification, yet do not express detectable aberrant HER-2 transcripts nor exhibit evidence of gene rearrangement (Hung et al., 1992; Hollywood & Hurst, 1993; Karlan et al., 1994; Jones et al., 1994). The PCR amplified the same size fragment of 312 bp from normal and from carcinoma cell line DNA (Fig. 6). These results indicate that the alternative 3'UTR sequence is contiguous with HER-2 3' terminal exon sequence in the human genome, demonstrating that the aberrant transcript is a result of alternative RNA processing, due to read-through of one and possibly two consensus polyadenylation signals (see underlined poly-A sites in Fig. 3). Therefore, the 8 kb mRNA is a direct product of the "wild-type" HER-2 oncogene rather than a product of gene rearrangement.

The alternative 8 kb HER-2 transcript displays increased stability in SKOV-3 cells. To investigate possible mechanisms for abundance of the alternative HER-2 transcript in SKOV-3, we measured its decay rate in comparison with the 4.5 kb transcript. Northern blot analysis was conducted on cells in culture treated with a-amanitin, an inhibitor of transcription (Fig. 7A). The data points were quantitated by phosphorimager analysis, averaged and plotted, and extrapolation of the calculated slope of the best fit line was used to estimate half-life ($t_{1/2}$). These data demonstrate a $t_{1/2}$ for the 4.5 kb HER-2 transcript of ~5.5 h, which is consistent with previously published determinations in SKOV-3, BT474, and SKBR-3 carcinoma cell lines (Karlan et al., 1994; Jones et al., 1994).

However, a significantly greater $t_{1/2}$ of approximately 13 h ($p<0.01$) was obtained for the 8 kb HER-2 transcript (Fig. 7B). A similar result was obtained when an additional inhibitor of transcription, actinomycin D, was employed (data not shown).

DISCUSSION

Although HER-2 gene amplification is a common mechanism for overexpression, many human tumors and cell lines display HER-2 mRNA and protein overexpression in excess of the level accountable by gene copy number, or in the absence of gene amplification (Hollywood & Hurst, 1993). The model ovararian carcinoma cell line, SKOV-3, exhibits the highest level of p185HER-2 expression and exhibits additional mechanisms for upregulation in addition to gene amplification (Yu et al., 1993). Furthermore, in addition to excess expression of the 4.5 kb HER-2 transcript, SKOV-3 also express an abundant alternative HER-2 transcript of 8 kb (Karlan et al., 1994; Lichtenstein et al., 1990; Kornilova et al., 1992; Jones et al., 1994).

Studies designed to repress HER-2 expression in SKOV-3 have targeted both HER-2 mRNA and protein, showing consistent downregulation of p185HER-2 (Juhl et al., 1997; Deshane et al., 1995; Weichen et al., 1995; Yu et al., 1993, 1995). Antisense oligodeoxynucleotides that targeting the HER-2 translation initiation sequence, which is shown here to be intact in the 8 kb transcript, effectively downregulate HER-2 mRNA and p185HER-2 by more than 90% in SKOV-3 cells (Weichen et al., 1995). Further, targeting the HER-2 mRNA in SKOV-3 cells with ribozymes also effectively down-regulated HER-2 protein expression (Juhl et al., 1997). Together with results presented here showing that the initiation sequence and overall coding sequence is intact and that the 8kb transcript is, under some conditions, 3-5 fold more abundant than the 4.5 kb transcript, supports the possibility that it may be translated. However, our findings do not directly show that this alternative transcript is translated into p185HER.

Our results further demonstrate that the 8 kb transcript does not result from a translocation event in SKOV-3, as previously suggested (Hung et al., 1992) and is, thus, a transcription product of an unaltered HER-2 gene. Together, the results of 5'RACE and RT-PCR on 8 kb and 4.5 kb mRNA from SKOV-3 cells indicate that the

difference lies only in the extension of the 3'UTR in the 8 kb transcript. PCR analysis of normal genomic DNA revealed that the novel extended 3'UTR sequence in the 8 kb transcript is adjacent to the terminal HER-2 exon in the genomic sequence. Therefore, the 8 kb transcript occurs by read-through of one and possibly two consensus polyadenylation signals.

Similarly, an alternative transcript of the EGFR of 10.6 kb has been found in A431 carcinoma cells (Lin et al., 1984) and in human glioblastomas (Sugawa et al., 1990), which both have EGFR gene amplification. This alternative EGFR transcript is reported to result from 3'UTR extension. The significance of this alternative transcript to expression of EGFR has not been characterized.

Significantly, we found that the 8 kb HER-2 transcript displays greater than two-fold increased half-life relative to the 4.5 kb transcript, suggesting that the 8 kb HER-2 is a stable, actively translated transcript, and likely does not contain nonsense mutations, which are known to destabilize mRNA (Beelman & Parker, 1995; Sachs, 1993). The 8 kb transcript had an estimated half-life of 13 hours compared to ~5.5 hours for the 4.5 kb HER-2 transcript, which is consistent with previous reports of approximately 6 hours for the 4.5 kb transcript in SKOV-3 cells (Karlan et al., 1994; Jones et al., 1994). Intrinsically altered HER-2 mRNA stability has not previously been reported. Since the coding region and 5'UTR are similar in the 8 kb and 4.5 kb HER-2 transcripts, it is likely that the extended 3'UTR of the alternative 8 kb HER-2 transcript confers its increased stability. The 3'UTR is known to contain sequences that control cytoplasmic mRNA functions, including stability, translation, and localization (Beelman & Parker, 1995; Jackson & Standart, 1990; Sonnenburg, 1994; Tanguay & Gallie, 1996; Sachs, 1993). Altered mRNA structural stability can result from changes affecting the length of the 3'untranslated region (3'UTR), sequences within the 3'UTR that confer RNA stabilization, including altered stem-loop structures, and/or introduction of binding sites for stabilizing proteins (Beelman & Parker, 1995; Jackson & Standart, 1990; Sonnenburg, 1994; Tanguay & Gallie, 1996; Sachs, 1993). Increased mRNA stability can lead to accumulation of message and, thus, increased translational capacity, resulting in protein overexpression. As observed previously (Yu et al., 1993), an increase in p185HER-2 is associated with a more metastatic phenotype and, thus, expression of the 8 kb message may confer a selective advantage for these aggressive ovarian carcinoma cells in a micrometastatic environment.

Conclusions

This work describes the characterization of an alternative 8 kb HER-2 mRNA. The 8 kb alternative mRNA is transcribed from an unaltered HER-2/neu gene, but by a different mechanism. This transcript is produced by DNA polymerase II read- through of a consensus polyadenylation signal in the terminal HER-2 exon (ch. 3, Figs. 3 & 6) Further, a second consensus polyadenylation site was found in the sequence of the extended 3'UTR that was also not utilized in these cells. The characterization of the 8 kb transcript resulted in determination of additional sequence that extends the known HER-2 gene sequence boundaries, defines the location of additional polyadenylation signals, and suggests an alternative mechanism for enhanced levels of HER-2 expression in human cancer.

8 kb HER-2 mRNA Expression

The aggressive ovarian cancer cell line, SKOV-3 expresses the 8 kb HER-2 transcript even more abundantly than the conventional 4.5 kb. Few other ovarian cell lines overexpress HER-2. Although the 8 kb HER-2 mRNA has not been found in any of these, it could be below the levels of detection. It is also possible that the 8 kb mRNA is expressed in ovarian cancers, since few primary human ovarian tumors have been examined at the mRNA level. While we have been unable to detect the 8 kb transcript in Northern analysis of fetal tissues, it could again be expressed below the levels of detection. Therefore, although no additional tissues or cell lines have been identified that express an 8 kb alternative transcript, we cannot yet rule out its presence in other cell lines and tissues.

Is the 8 kb mRNA translated?

Investigations of HER-2 protein products in SKOV-3, which express abundant amounts of the 8 kb transcript, have revealed no alternative protein product resulting from this unusually large transcript. Rather, it appears that selection for expression of the 8 kb mRNA is based on its enhanced intrinsic stability. That the 8 kb HER-2 mRNA is both polyadenylated and exhibits increased stability suggest that it could be translated into p185HER-2. Untranslated mRNAs, or mRNAs with premature translation termination, have been shown to be unstable (reviewed in Surdej et al., 1994).

Future studies should be directed toward determining whether the 8 kb transcript is translated. A polysome profile could be conducted as

an assessment of translation initiation rate. Furthermore, a polysome profile comparing the 8 kb and 4.5 kb transcripts would indicate whether the extended 3'UTR of the 8 kb might facilitate translation. Evidence suggests that sequences or secondary structure of the 3'UTR in mRNA, by providing sites for RNA-binding protein association, might regulate rate of translation initiation by facilitating interactions between poly(A)-binding protein (PABP) and the 5'methyl-7-guanine cap structure (Hake & Richter, 1997; Jackson, 1993). Another method would be *in vitro* translation of the transcript, from an expression vector construct containing the cDNA sequence of the entire 8 kb transcript.

Additionally, to test whether the 8 kb transcript is translated, the cDNA could be transfected as a mammalian expression vector into a cell line that lacks HER-2 expression, such as Ba/F3. Further studies of the expression of the 8 kb with that of the 4.5 kb expression could be conducted to compare the intrinsic stability and translation of these two transcripts.

Possible Functions of the 3'UTR Extension in the 8kb mRNA

The 3'UTR, like the coding region of mRNA, contains encrypted information. In contrast to coding sequence, the complex codes within 3'UTR determine precise regulation of gene expression and have yet to be completely deciphered. Some of the information may be structural, involving RNA secondary structure determined by primary sequence of the 3'UTR, which creates specific protein binding sites. The hexanucleotide AAUAAA has long been recognized as a nuclear polyadenylation signal, but we are just beginning to recognize common sequence elements that confer mRNA stability, cytoplasmic poly(A) extension, subcellular localization, translation initiation, and rapid degradation.

Perhaps the most thoroughly studied of these functions is 3'UTR-mediated mRNA stability. Sequence elements have been identified, acting in cis within the 3'UTR, that confer rapid degradation of some mRNAs (Asson-Baters et al., 1994; Shaw & Kamen, 1986). Other elements may confer stability, but these appear to be more complex and specific to RNA-protein interactions (Zhou et al., 1998; Jackson, 1993). The overall emerging theme is that mRNA stability can be conferred by properties that are determined by the secondary structure within its 3'UTR. This may involve stabilizing stem-loop structural formations or binding of an mRNA stabilizing protein.

The expression of the 8 kb HER-2 is reminiscent of developmental or maternal mRNAs which sometimes differ in 3'UTR length and/or sequence from that of their adult counterparts (Hake & Richter, 1997). The difference in 3'UTRs between the 4.5 and 8 kb HER-2 transcripts might confer an altered rate of nuclear export, subcellular localization and/or temporal expression (Jackson, 1993). Although neither of the known elements that confer such regulation, a 60 nt sequence (Braun, 1991) and the translation inhibition element (TIE)(Robbie, 1995), were found in the 8 kb 3'UTR sequence obtained in a fractionated cDNA library clone, the clone was incomplete. Therefore, these possibilities have not yet been completely investigated.

Implications and Future Studies of 8 kb HER-2 mRNA

The expression of the 8 kb alternative HER-2 mRNA demonstrates regulation of polyadenylation site choice. Because this transcript contains two skipped consensus hexanucleotide sequences within its 3'UTR, it could provide a means for studying the mechanism regulating nuclear polyadenylation site choice and mapping the sequences that might confer this process. Such regulatory mechanisms may be important in developmental gene expression.

It would be of interest to investigate whether selective deletion of the 8 kb HER-2 transcript alters the potent oncogenicity of SKOV-3. Possible methods include the use of antisense oligonucleotides or ribozymes designed to specifically target the extended 3'UTR. For oligos, depending on the region of 3'UTR to which they are complimentary, the resulting effect could be further stabilization of the 8 kb. Therefore, 8 kb mRNA stability would need to be assessed in response to an array of antisense oligos. Use of ribozymes is an alternative method of selectively targeting the 8 kb transcript for degradation. The growth of SKOV-3 in soft agar or invasive behavior could be assayed to score the effects of altering 8 kb HER-2 expression. An alternative method to assess the function of the 8 kb HER-2 transcript would be to transfect its cDNA into exogenous cells. Thus, an elaborate investigation of the behavior of cells, in which expression of the 8 kb HER-2 transcript is controlled, might elucidate its function.

In summary, the current model of HER-2 oncogenic potency needs revision to incorporate important alternative forms of the HER-2 gene products that we have characterized in the Clinton laboratory.

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APPENDIX

Fig. 1. SKOV-3 cells overexpress a HER-2 transcript of 4.5 kb and 8 kb, and p185HER-2/*neu*. The left panel, **A**, shows a Northern blot of mRNA from T47D, NIHOVCAR3, CAOV3, and SKOV-3 (2.5 µg/lane), hybridized with a 5'HER-2 cDNA probe (top) and a β-actin cDNA probe (bottom) as a loading control. The right panel, **B**, is a western blot of 20 µg protein from each cell line reacted with antibodies against the C-terminus of p185HER-2 (28).

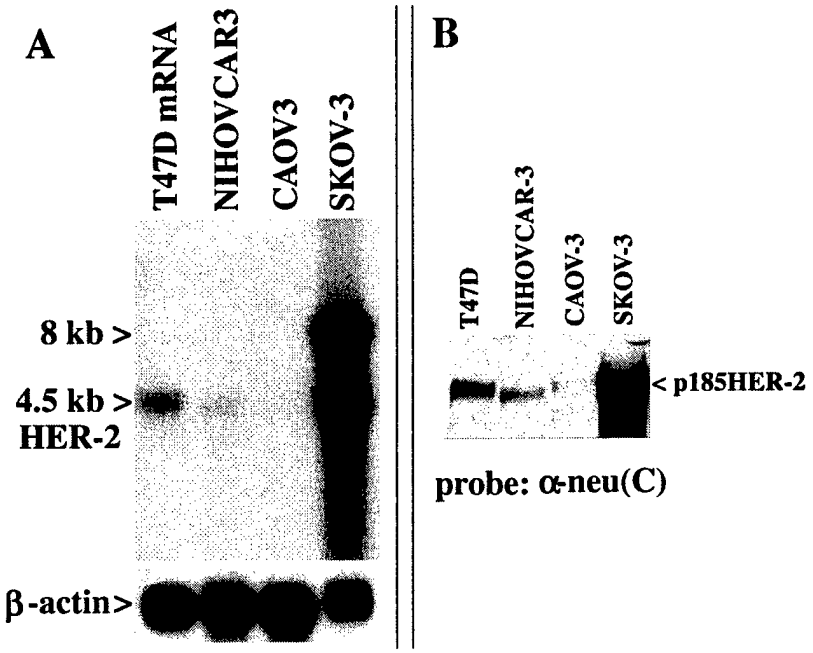

Fig. 2. The 5'UTR and coding sequence of the 8 kb HER-2 transcript are similar to that of the 4.5 kb 'wild-type' transcript, but the 8 kb transcript differs in its 3'UTR. **A:**PCR amplification products from the 8 kb fraction, 4.5 kb fraction, p9002 plasmid containing HER-2 cDNA sequence, and a control without DNA. The right panel is a Southern blot of the gel hybridized with a HER-2 5' coding region-specific probe (described in Methods). **B:** The left panel is an ethidium bromide-stained 2% agarose gel of PCR products from 5' RACE conducted on reverse transcribed SKOV-3 mRNA from the 4.5 kb fraction, the 8kb fraction; and no DNA (control). The right panel is a Southern blot hybridized with a oligonucleotide probe, end-labelled with T4 kinase, which is identical to nucleotides 145-163 of the HER-2 cDNA (1). **C:** The left panel shows 3' RACE products from the 4.5 kb fraction, 8 kb fraction, T47D cDNA, the control plasmid, p9002, and no DNA control. The right panel is a Southern blot of this gel, hybridized with a ^{32}P- labelled HER-2 3'UTR-specific oligonucleotide identical to nt 3918-3943 of HER-2 cDNA.

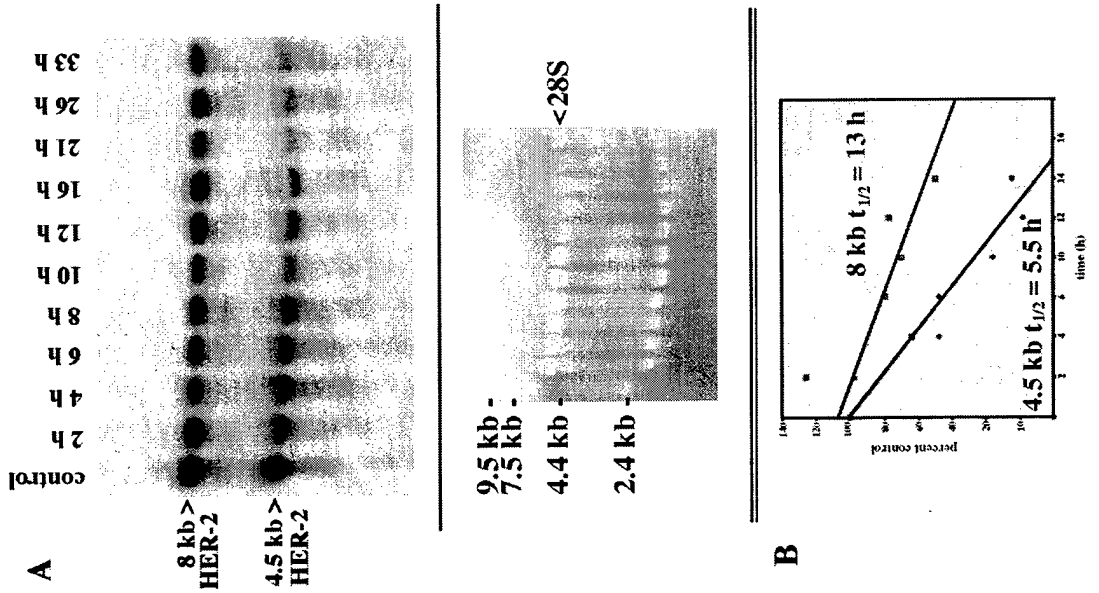

Figure 2C

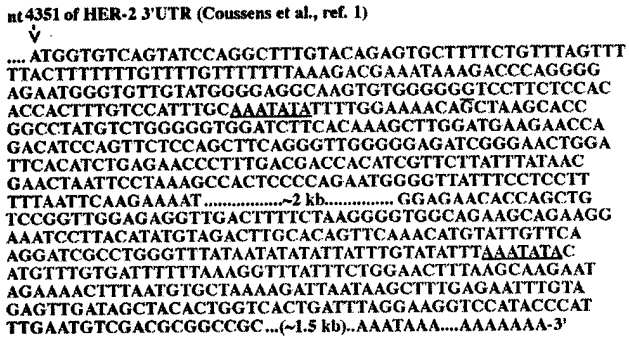
nt 4351 of HER-2 3'UTR (Coussens et al., ref. 1)
.... ATGGTGTCAGTATCCAGGCTTTGTACAGAGTGCTTTTCTGTTTAGTTT
TTACTTTTTTTGTTTTGTTTTTTTAAAGACGAAATAAAGACCCAGGGG
AGAATGGGTGTTGTATGGGGAGGCAAGTGTGGGGGGTCCTTCTCCAC
ACCACTTTGTCCATTTGCAAATATATTTTGGAAAACAGCTAAGCACC
GGCCTATGTCTGGGGGTGGATCTTCACAAAGCTTGGATGAAGAACCA
GACATCCAGTTCTCCAGCTTCAGGGTTGGGGGAGATCGGGAACTGGA
TTCACATCTGAGAACCCTTTGACGACCACATCGTTCTTATTTATAAC
GAACTAATTCCTAAAGCCACTCCCCAGAATGGGGTTATTTCCTCCTT
TTTAATTCAAGAAAAT.....~2 kb..... GGAGAACACCAGCTG
TCCGGTTGGAGAGGTTGACTTTTCTAAGGGGTGGCAGAAGCAGAAGG
AAATCCTTACATATGTAGACTTGCACAGTTCAAACATGTATTGTTCA
AGGATCGCCTGGGTTTATAATATATATTATTTGTATATTTAAATATAC
ATGTTTGTGATTTTTTAAAGGTTTATTTCTGGAACTTTAAGCAAGAAT
AGAAAACTTTAATGTGCTAAAAGATTAATAAGCTTTGAGAATTTGTA
GAGTTGATAGCTACACTGGTCACTGATTTAGGAAGGTCCATACCCAT
TTGAATGTCGACGCGGCCGC...(~1.5 kb)..AAATAAA....AAAAAAA-3'

Fig. 4. Sequence-specific hybridization of the novel 3'UTR sequence with 8kb mRNA. Northern blot of T47D (lanes 1, 3) and SKOV-3 (lanes 2, 4) mRNA (2.5 μg/lane), hybridized with 5'HER-2 cDNA (left panel) or unique sequence obtained from the 8 kb cDNA clone (right panel).

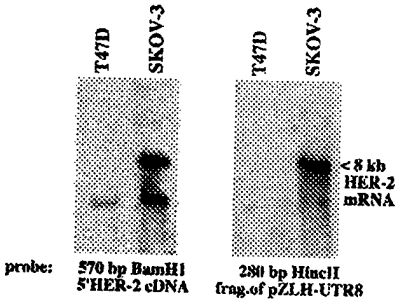

Fig. 5. SKOV-3 cells exhibit EcoR1 restriction fragment length polymorphism in the HER-2 gene. 10 μg of genomic DNA from either SKOV-3 or BT474 cells was digested with excess amounts of restriction enzymes for 24 h at 37° C. Digested DNA was resolved in 0.8% agarose gels, transferred to membrane in 0.4 N NaOH, and hybridized with 10^7 cpm of random-primed ^{32}P-labelled HER-2 cDNA. The blot was washed at high stringency and subjected to phosphorimage analysis.

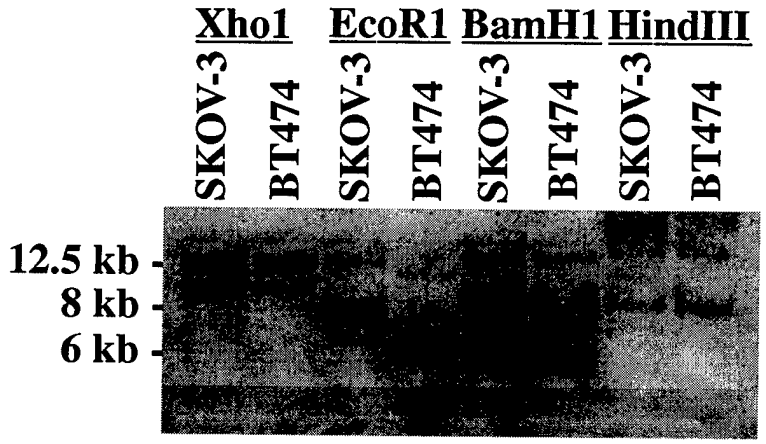

Fig. 6. Unique 3'UTR sequence contained within the 8 kb HER-2 transcript is contiguous with HER-2 terminal exon sequence in the genome. Ethidium bromide-stained 1.5% agarose gel of PCR products amplified from genomic DNA of SKOV-3, BT474, IOSEVAN, and normal human blood, or no DNA template. A 312 bp product was amplified from all four DNA templates (lanes 1-4), which is the expected size if the unique 3'UTR sequence from the 8 kb transcript is contiguous with the HER-2 exonic 3'UTR sequence in the genome.

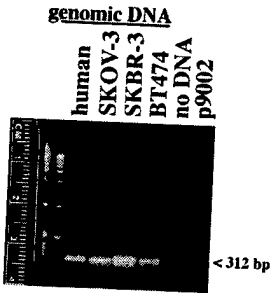

Fig. 7. The alternative 8kb HER-2 transcript displays increased stability. **A:** The top panel shows a Northern blot of SKOV-3 total RNA (10 µg/lane) extracted at the indicated time points after addition of "-amanitin (24 µg/ml) and hybridized with a HER-2 cDNA probe. The bottom panel shows the 28S RNA in the same gel prior to transfer. **B:** "Best fit line" of data points from five independent experiments. The half-life for each transcript was obtained by extrapolation of 50% of control signal at time "0" or of untreated cells.